VILNIUS UNIVERSITY
STATE RESEARCH INSTITUTE
CENTRE FOR INNOVATIVE MEDICINE

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BIOMARKERS OF BACTEREMIA AND SEPSIS IN PEDIATRIC ONCOLOGY PATIENTS WITH FEBRILE NEUTROPENIA

Summary of doctoral thesis
Biomedical sciences, medicine (06 B);
Immunology, serology, transplantation (B 500)

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This study was carried out in 2008 - 2012 at the Institute of Immunology of Vilnius University and, after reorganization, at the State Research Institute Centre for Innovative Medicine

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VILNIAUS UNIVERSITETAS
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INOVATYVIOS MEDICINOS CENTRAS

Vincas Urbonas

VAIKŲ, SERGANČIŲ NAVIKINĖMIS LIGOMIS, BAKTERIEMIJOS BEI SEPSIO BIOŽYMENYS FEBRILINĖS NEUTROPENIJOS EPIZODO METU

Daktaro disertacijos santrauka
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Introduction

During the last decades more advanced treatment methods such as a myelosupressive therapy, immunotherapy, transplantation of haemopoetic stem cells have significantly increased the survival rate of oncologic patients. A common consequence of modern chemotherapy is a decreased number of neutrophils and other fast replicating cells due to toxicity. Chemotherapy-induced neutropenia is one of the main side effects of oncology patients' treatment. Fever (characterized as an axillary temperature of more than 38,5°C) can be the first and often single sign of bacterial infection. Other classical features of inflammatory process are often reduced in cancer patients with neutropenia due to an impaired immune response. Consequently, patients with chemotherapy-induced febrile neutropenia (FN) require careful attention. An empiric usage of intravenous broad-spectrum antibiotics was introduced in 1971 and resulted in a dramatic decline in morbidity and mortality from bacterial infections. However, 70–89% of patients with febrile neutropenia who received standard antimicrobial treatment had no causative bacteria in their blood cultures. Also, results of microbiological evaluation usually can be available only 2 or 3 days later and they cannot help doctor to make a therapeutic decision at the time of the patient's admission to the hospital. Besides, a wide range of bacteria, febrile neutropenia in these patients can be originated from viruses, malignancy itself, usage of chemotherapeutic drugs, mucosal damage or transfusions of blood products. Patients without documented clinical and microbiological evidence for a bacterial infection usually have a shorter duration of fever and lower risk for clinical complications development. Of note, usually children with chemotherapy related febrile neutropenia are hospitalized and treated with intravenous broad spectrum antimicrobial agents and they are eligible for discharge when they become afebrile and the absolute neutrophil count has recovered to more than $0{,}5 \times 10^{9}$/l. This approach leads to over-treatment of a substantial number of patients without confirmed bacterial infection and as a consequence of it, both the risk of bacterial resistance and the costs of health care increase accordingly, whereas quality of life for these patients and their families decreases. Patients at

low risk for infection are potential candidates for ambulatory treatment. Therefore, re-evaluation of current approach in terms of new treatment and diagnostic strategies for the patients with FN is under way, with the focus on diagnostic biomarkers for the determination of a group of the patients with a low risk for the bacterial infection are under investigation and of great interest.

The aim of the dissertation:

This study was designed to evaluate the response of innate immunity to acute bacterial inflammation in terms of cytokines and other molecules concentration changes in the blood of investigated childhood oncology patients during the beginning of febrile neutropenia episode and to assess the relevance of these biomarkers for sepsis/bacteremia evaluation.

The objectives of the dissertation were as following:

1. Determination of interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and soluble interleukin-2 receptor (sIL-2R) concentrations in the blood and evaluation of these biomarkers for the feasibility of sepsis/bacteremia diagnostic, screening and monitoring purposes;
2. Determination of other biomolecules such as C-reactive protein (CRP), procalcitonin (PCT) and soluble human leukocyte antigen-G (sHLA-G) concentrations in the blood and evaluation of these biomarkers for the feasibility of sepsis/bacteremia diagnostic, screening and monitoring purposes.

Scientific novelty

In Lithuania investigation of early biomarkers of bacterial infection in childhood oncology patients with FN has not been carried out. Overseas cytokines and other biomarkers have been evaluated in above mentioned settings, however, a comprehensive and systematic assessment of cytokines and other biomolecules involved in pathogenesis of acute bacterial inflammation have not been performed. In this study we evaluated seven biomarkers (IL-6, IL-8, IL-10, sIL-2R, CRP, PCT, sHLA-G) involved in the pathogenesis of acute bacterial inflammation in childhood oncology patients with FN and systematically assessed them for the suitability of bacteremia/sepsis evaluation.

The defensive statements

1. Assessed cytokines, cytokines receptors participate in pathogenesis of acute bacterial inflammation and they can be used as a diagnostic tool for the sepsis/bacteremia evaluation;
2. Changes in concentrations of CRP, PCT, sHLA-G in the blood can be used as diagnostic biomarkers for the evaluation of acute bacterial inflammation.

Materials and methods

Patients and definitions. This study was performed at Vilnius University Children Hospital and State Research Institute Centre for Innovative Medicine from 2009 to 2011. Serum samples were collected during 82 fever episodes in a total of 53 oncology patients, including acute lymphoblastic leukemia (n=32), acute myeloblastic leukemia (n=4), non-Hodgkin's lymphoma (n=2) and non-hematologic malignancies (n=15), respectively. The study population consisted of pediatric oncology patients admitted to the hospital with the diagnosis of neutropenia and fever. All patients underwent treatment with cytotoxic chemotherapy. There were 23 females and 30 males with a median age of 6 years (range 1–17 years). An informed consent, after verbal and written information provision, was obtained from all patients. Permission for this study was provided by the Lithuanian Committee of Bioethics (Nr.158200-12-130-35). Neutropenia was defined as an absolute neutrophil count (ANC) of less than $0,5x10^9$/l at the onset of fever. Fever was described as an axillary body temperature of more than 38,5°C in one measurement. None of the included patients were administered antibiotics before enrolment. Clinically documented sepsis was defined as a core temperature of more than 38,5°C, tachycardia, clinical syndrome associated with a high probability of infection (chest radiograph consistent with pneumonia, petechial, purpuric rash, or purpura fulminans) with or without one or more organ dysfunction. Bacteremia was defined as a microbial growth in one of the blood culture bottles. For coagulase-negative *Staphylococcus* species, two positive blood cultures from different sites were required, otherwise the result was considered as possible contamination.

According to microbiological and clinical findings, patients with episodes of febrile neutropenia were classified into 2 groups: 1) fever of unknown origin (FUO) group – patients with negative blood culture, absence of clinical signs of sepsis and clinically or microbiologically documented local infection, 2) septic/bacteremia (SB) group – patients with positive blood culture (documented Gram-positive or Gram-negative bacteremia) and/or clinically documented sepsis.

Sampling and laboratory analysis. Venous blood samples for the interleukins and other biomarkers determination were collected into 5 ml Vacutest® polypropylene tubes with K3 EDTA (Kima Company, Italy). The tubes were centrifuged at 2000 g for 10 minutes to separate the plasma and separated plasma was stored in Eppendorf tubes at -20 °C until evaluated. The first blood sample was taken on admission (day 1) then FN was confirmed to the patient and before antimicrobial treatment started. Remaining samples were taken on day 2 and day 3, respectively. Concentrations of IL-6, IL-8, IL-10 and sIL-2R in the plasma of patients were measured by chemiluminescence immunoassay using automated random access analyzer Immulite (Siemens Healthcare Diagnostics, Deerfield, IL, USA). Concentrations of PCT were evaluated by enzyme-linked fluorescent immunoassay using automated analyzer Vidas (Biomerieux, France). sHLA-G was measured using an ELISA assay (Exbio Praha) and CRP was evaluated by turbidimetric assay using automated random access analyzer Cobas Integra 400 (Roche Diagnostics, Switzerland).

Venous blood samples for one set of blood cultures (aerobic, anaerobic) were aseptically collected into separate vials, which were incubated in the Bactec 9240 incubator (Becton Dickinson, USA). Identification of microorganisms was performed by standard microbiological methods. All samples for the microbiological assessment were taken before the antimicrobial treatment started.

Statistics. Summary statistics were expressed as median. A comparison between groups of patients was carried out for all FN episodes using Mann-Whitney non-parametric test. Associations were estimated as statistically

significant when p value < 0,05. The diagnostic properties of biomarkers were evaluated by receiver-operating characteristic (ROC) analysis. This statistical method provides index of diagnostic accuracy of a test, which named area under the ROC curve (AUC). AUC is proportional to the probability of a correct distinction (1.0 means perfect discriminatory ability, 0,5 – no better than chance). Positive predictive value (PPV) and negative predictive value (NPV) were calculated for each cut-off level. One-way analysis of variance (ANOVA) was used to determine significant differences among the biomarkers of the same group through the first three consecutive days. Binary logistic regression was used to describe the relationship between two variables (confirmed bacteremia/sepsis and values of biomarkers). Statistical analysis was performed on data from FN episodes rather than individual patients. The commercial statistical software was used for calculations (MedCalc Software, version 11.4.2.0, Belgium and SPSS Software version 15.0.1, USA).

Results and discussion

All patients with FN undergo hospitalization and broad-spectrum antibiotic treatment, although this kind of approach is essential only in patients with bacterial infection. Therefore, early and reliable biochemical markers for bacterial infection are needed to avoid over-treatment with antibiotics and decreasing quality of life of patients due to unnecessary hospitalization.

In this present single-center prospective study we focused on 3 initial days of fever in patients with neutropenia and diagnostic value of 7 different biomarkers to discriminate patients with bacteremia/sepsis. After grouping the patients into two groups (FUO and SB), according to the results of bacteriological tests and clinical evaluation of the patients, we measured the levels of cytokines (IL-6, IL-8, IL-10), their receptors (sIL-2R) and other biomarkers (PCT, CRP, sHLA-G) for three consecutive days, if the number of absolute neutrophil count (ANC) was less than $0,5x10^9$/l and fever started (≥38,5^oC).

Clinically/microbiologically confirmed infection. BS group consisted of 22 patients, who had a total of 29 episodes of febrile neutropenia. Positive blood

cultures were obtained in 23 episodes. Gram-negative microorganisms were obtained in 12 cases *(Escherichia coli – 6, Klebsiella pneumoniae – 2, Bacteroides urealyticus – 1, Pseudomonas aeroginosa – 1, Enterobacter asburiae – 1, Klebsiella oxytoca - 1),* mixed microflora were detected in 3 episodes of febrile neutropenia (*Escherichia coli/Bacillus cereus, Staphylococcus haemolyticus/Klebsiella pneumoniae and Aeromonas hydrophila /Pseudomonas putida)* and Gram-positive microorganisms in 8 cases (*Staphylococcus hominis – 2, Staphylococcus epidermidis – 5, Clostridium tyrobutyricum -1*). Clinically documented sepsis was confirmed in 6 episodes of FN. Incidence of bacteremia/sepsis in our investigated group of children with FN was 35% and this rate is in line with results of other investigators, who stated that 10-45% of febrile neutropenic children in their investigated groups suffered from bacteremia/sepsis.

Performance of investigated biomarkers*.* The discriminatory power of cytokines and other biomarkers for bacteremia/sepsis identification in febrile neutropenic cancer patients was assessed according to AUC. This method summarizes the validity coefficients of a test and provides an overall index of diagnostic accuracy (the area under the ROC curve) from a plot of sensitivity against the false-positive rate (1 – specificity) for all possible cut-off values. Sensitivity, specificity, negative and positive predictive values (NPV, PPV) were also evaluated. The ROC curves and boxplots for evaluated biomarkers on day 1 are shown in Figures 1-7.

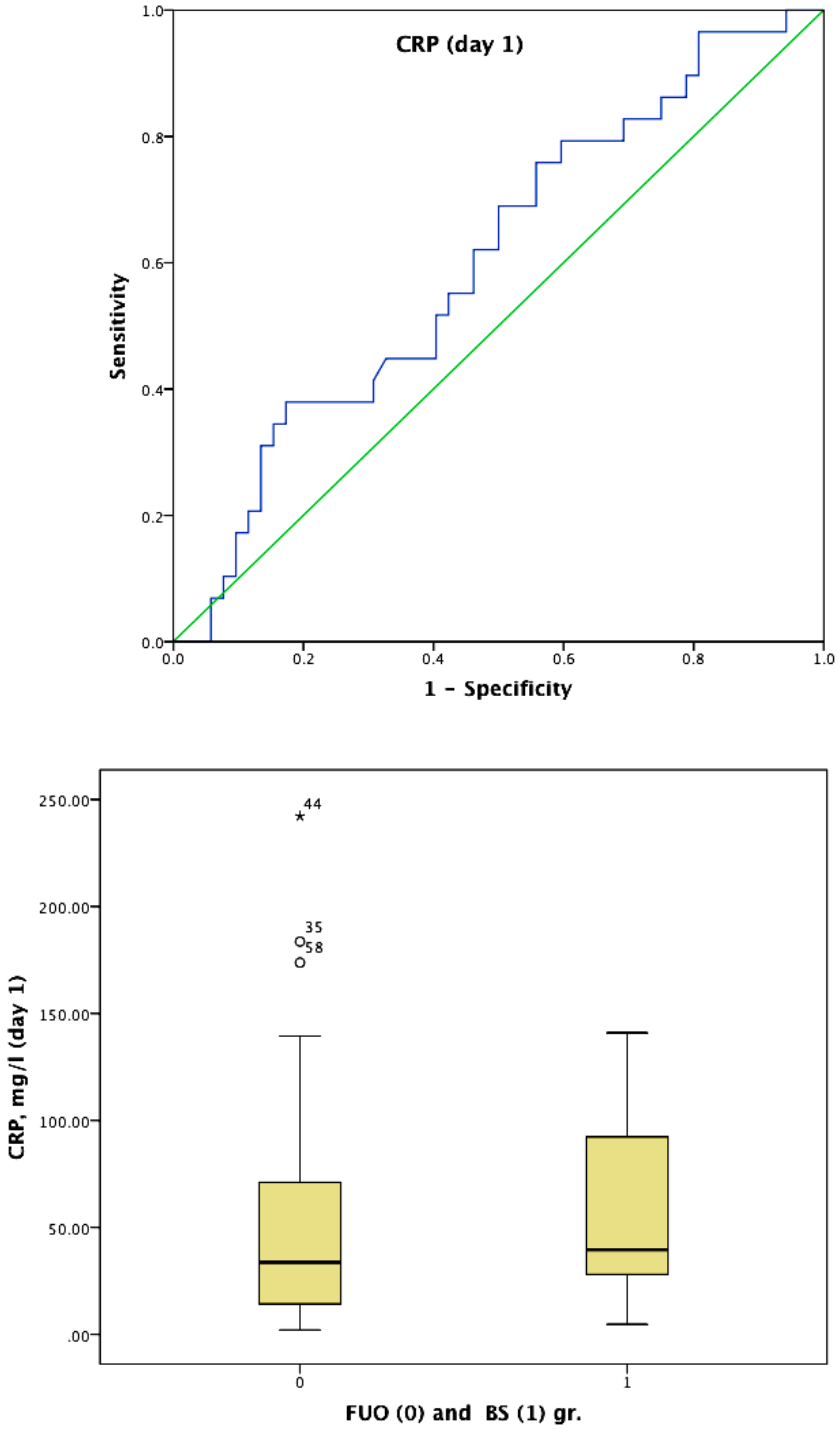


Figure 1. ROC curve and boxplot for CRP on day 1 (AUC=0,60; P=0,1229). Horizontal lines in interquartile box represent the median (lower part of the figure)

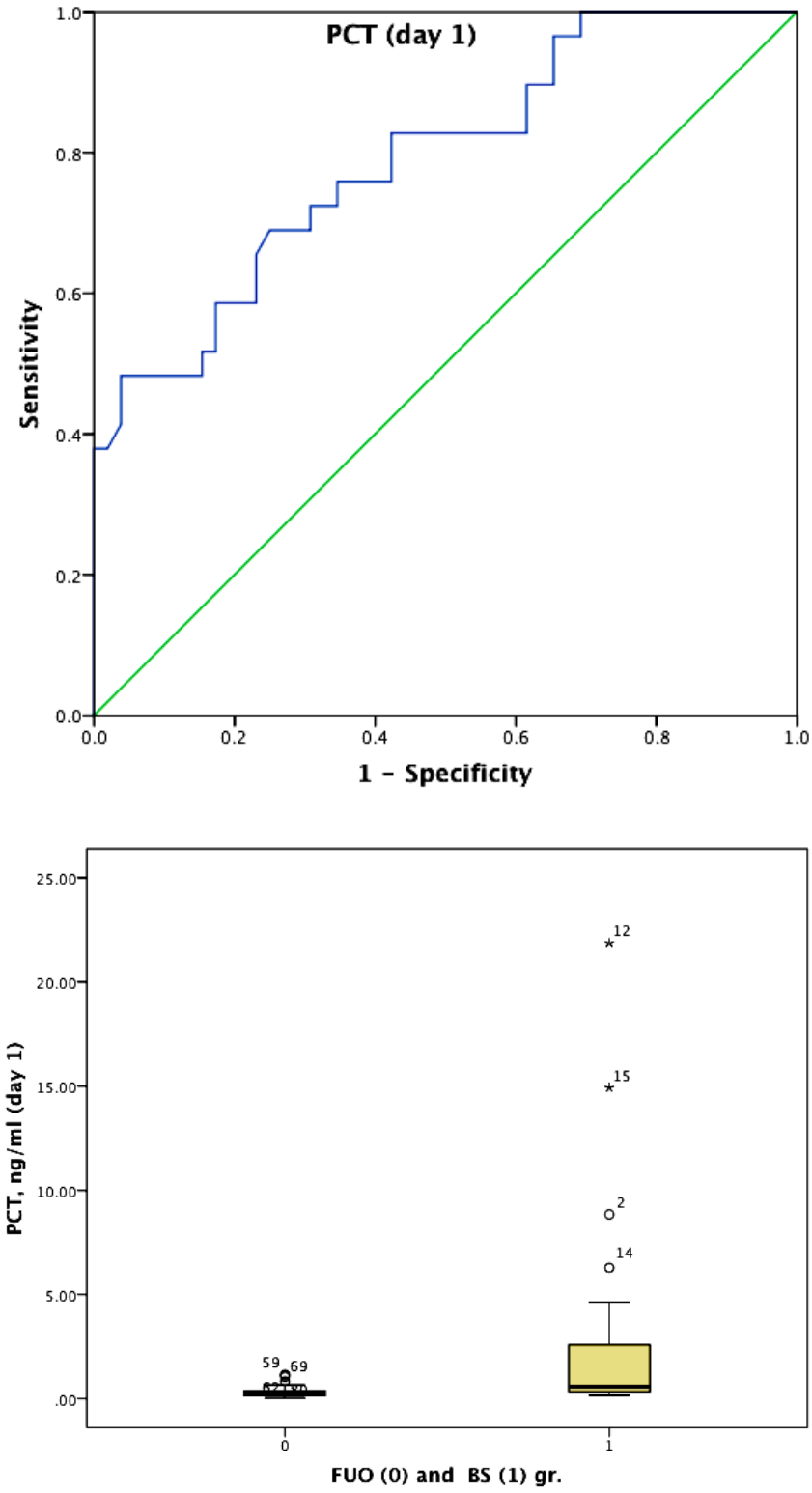


Figure 2. ROC curve and boxplot for PCT on day 1 (AUC=0,79; P<0,0001). Horizontal lines in interquartile box represent the median (lower part of the figure)

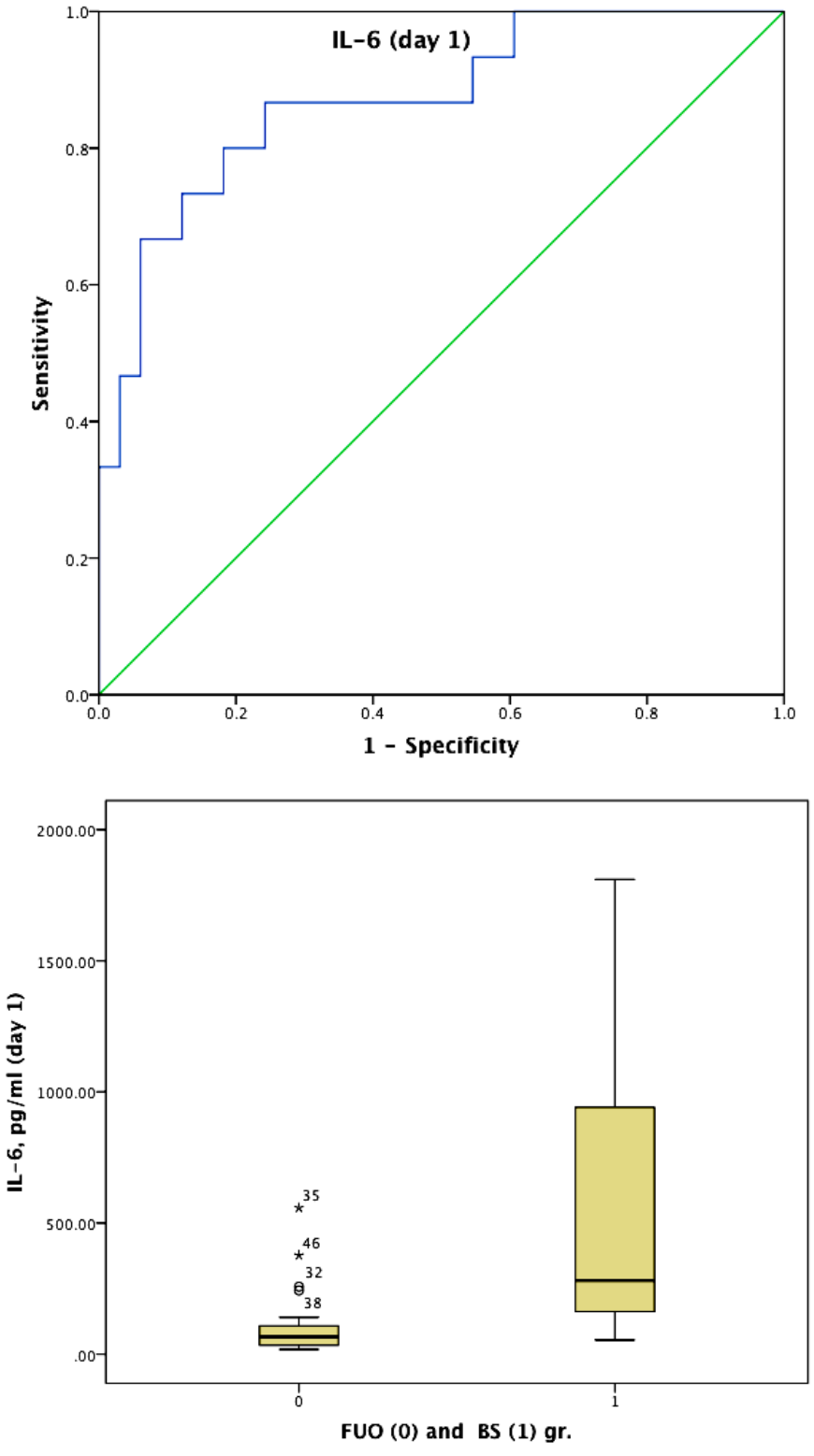


Figure 3. ROC curve and boxplot for IL-6 on day 1 (AUC=0,87; P<0,0001). Horizontal lines in interquartile box represent the median (lower part of the figure)

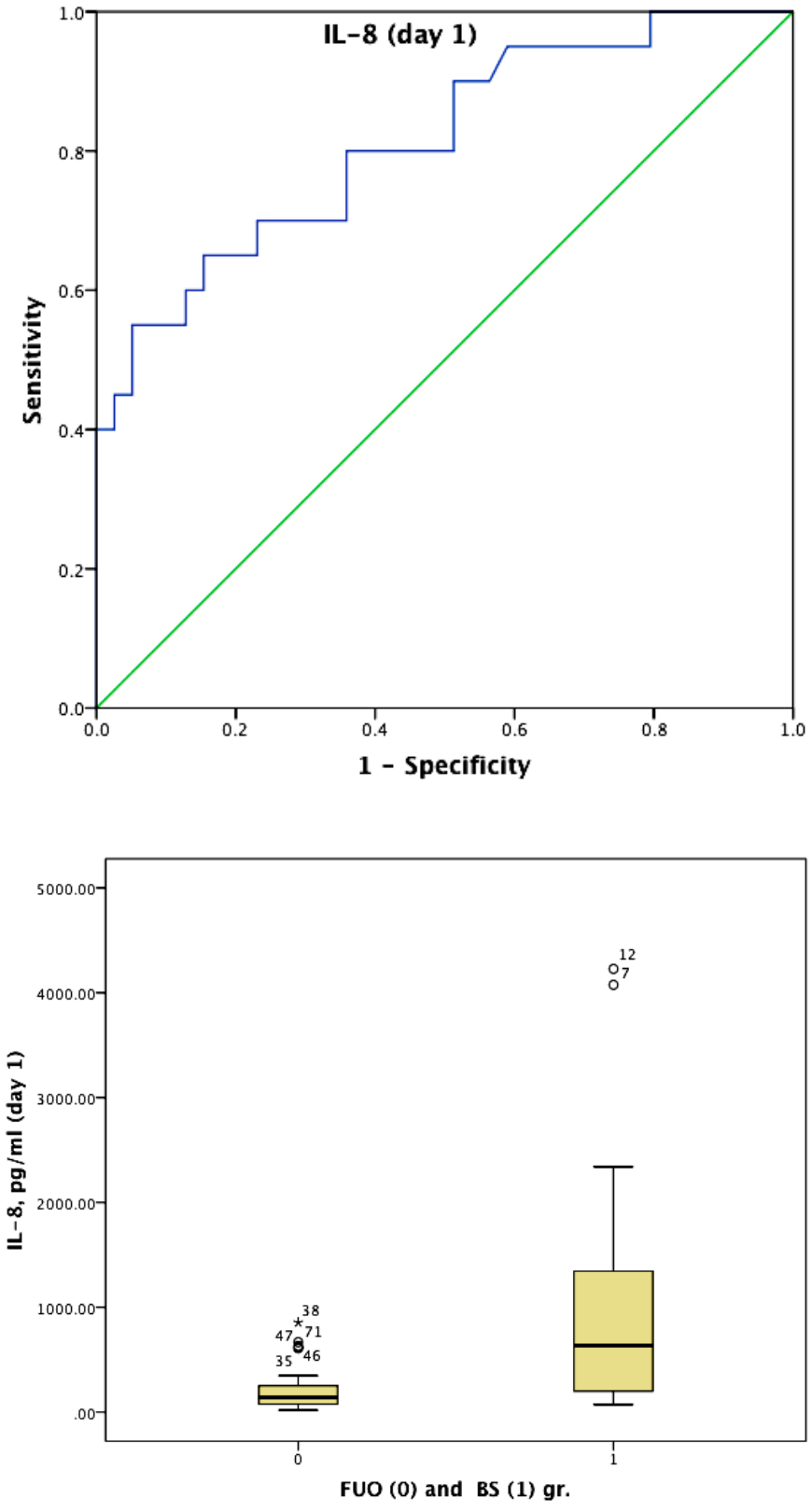


Figure 4. ROC curve and boxplot for IL-8 on day 1 (AUC=0,81; P<0,0001). Horizontal lines in interquartile box represent the median (lower part of the figure)

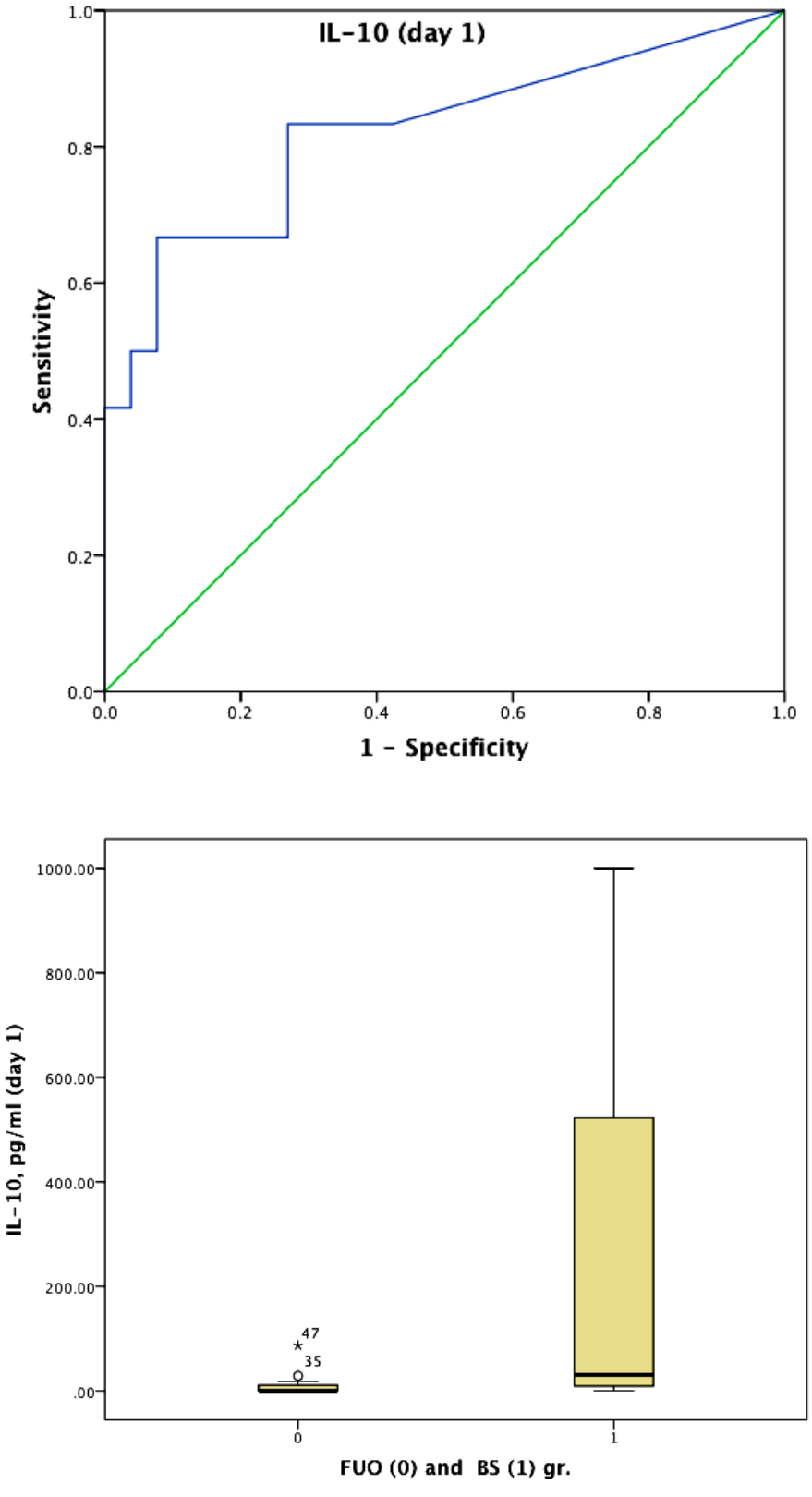


Figure 5. ROC curve and boxplot for IL-10 on day 1 (AUC=0,82; P=0,0001). Horizontal lines in interquartile box represent the median (lower part of the figure)

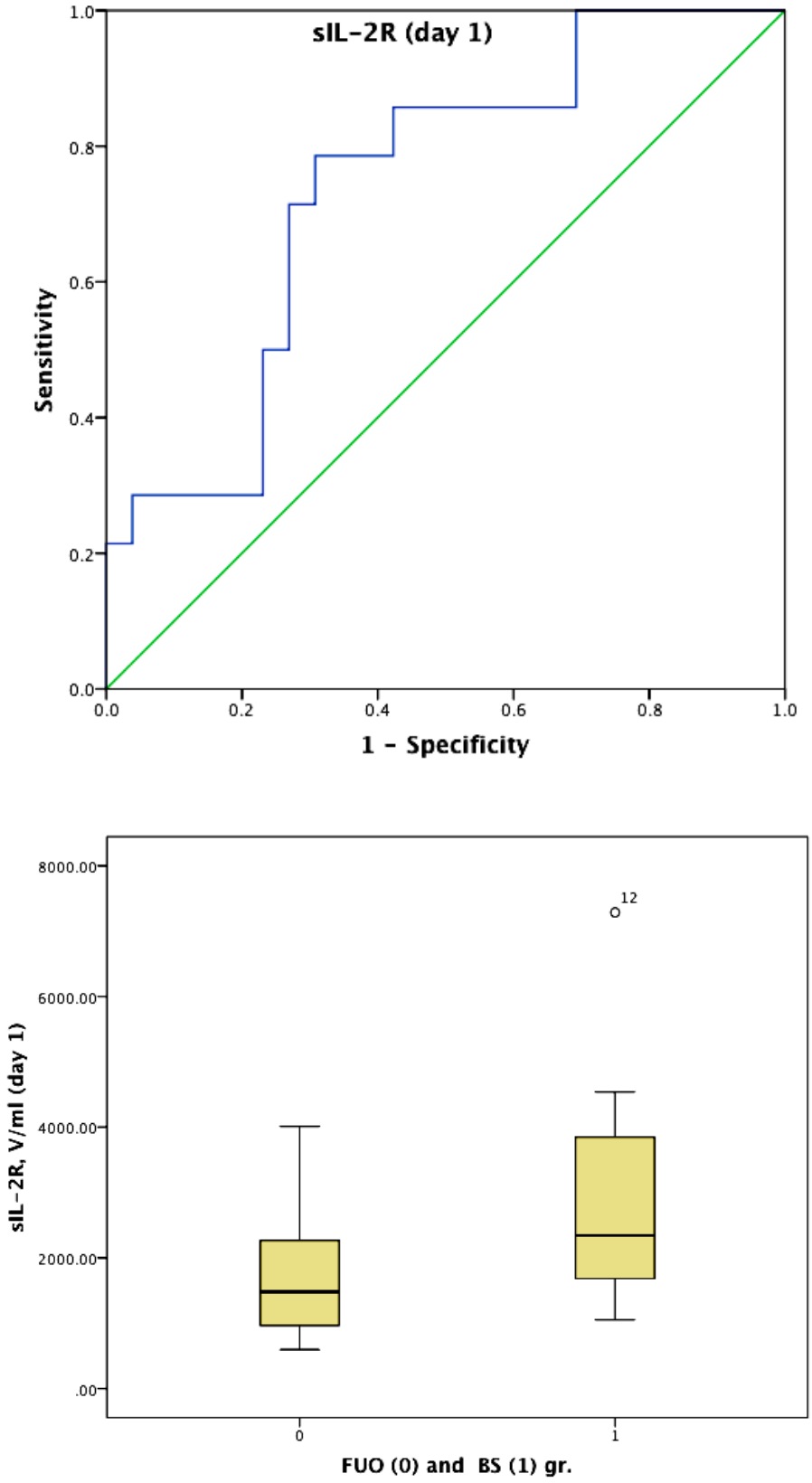


Figure 6. ROC curve and boxplot for sIL-2R on day 1 (AUC=0,74; P=0,0061). Horizontal lines in interquartile box represent the median (lower part of the figure)

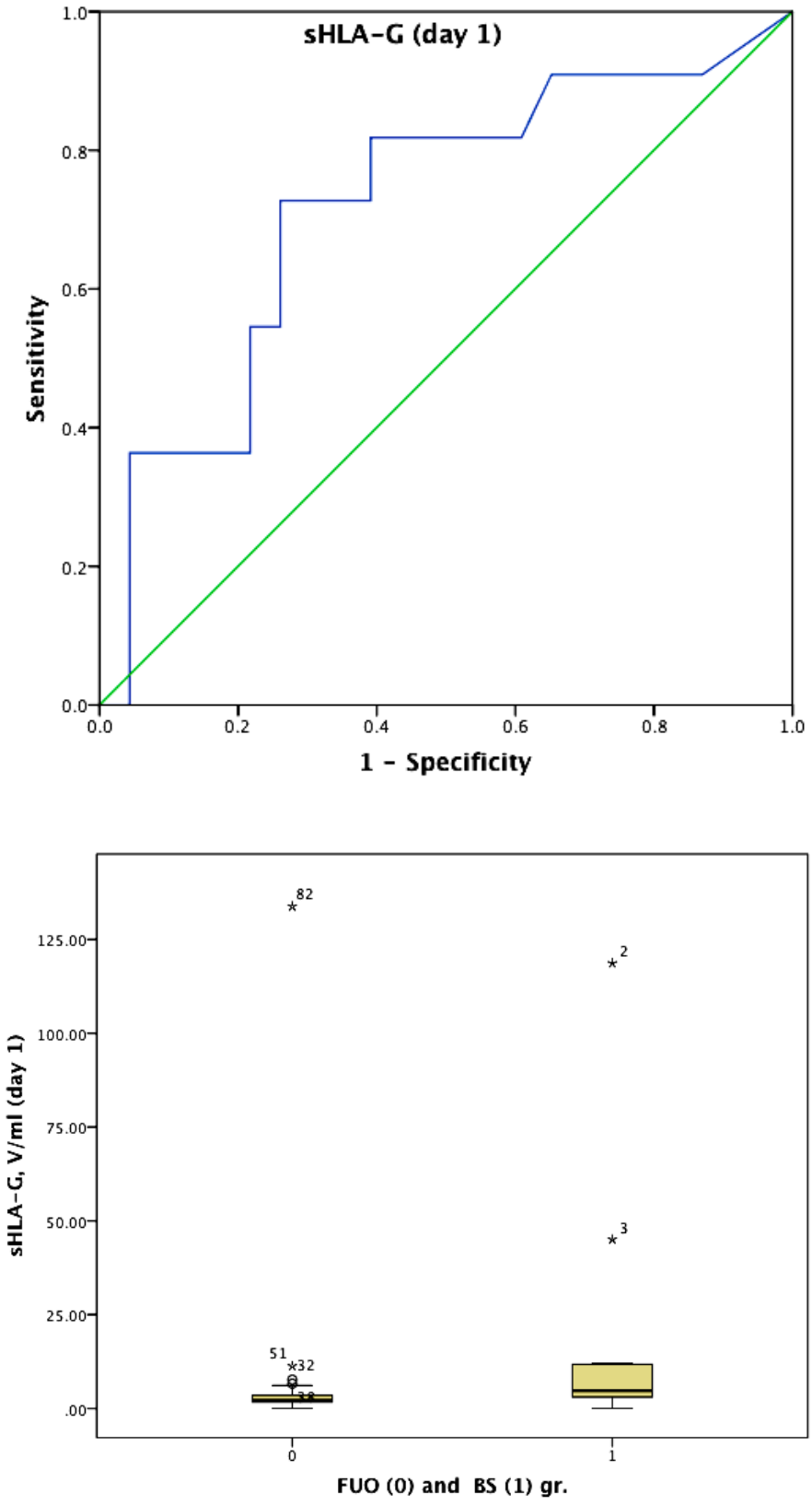
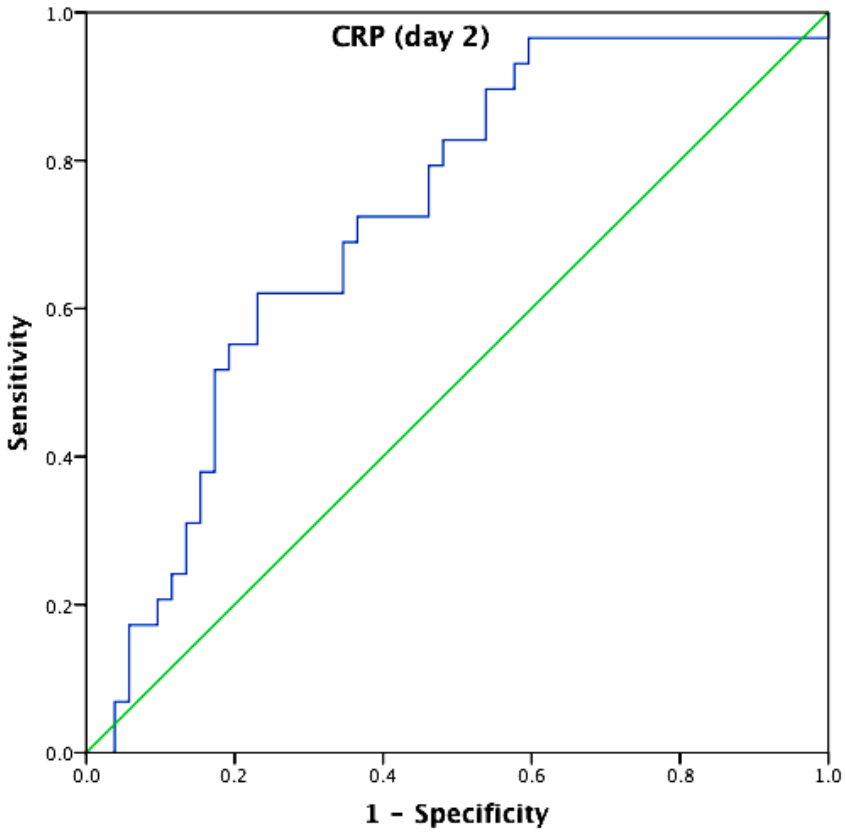


Figure 7. ROC curve and boxplot for sHLA-G on day 1 (AUC=0,72; P<0,0274). Horizontal lines in interquartile box represent the median (lower part of the figure)

The areas under the curves (AUC) for IL-6, IL-8 and IL-10 for day 1 were above 0,80 (AUC $_{IL-6}$=0,87; AUC $_{IL-8}$=0,81; AUC $_{IL-10}$=0,82) suggesting that these biomarkers discriminated bacteremia/sepsis with a good accuracy. AUC of other investigated biomarkers were lower than 0,80 on day 1 and they were in the range of 0,72–0,79 (AUC $_{sHLA-G}$=0,72; AUC $_{sIL-2R}$=0,74; AUC $_{PCT}$=0,79). AUC for CRP on day 1 was only 0,60 and it was not statistically significant (P=0,1229).

The ROC curves and boxplots for evaluated biomarkers on day 2 are shown in Figures 8 - 13.



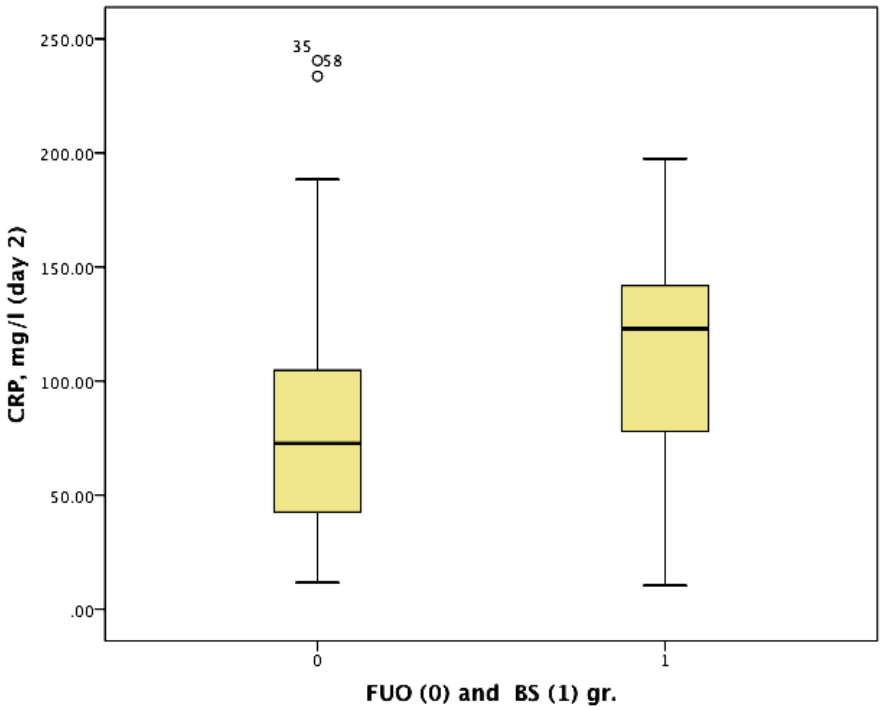


Figure 8. ROC curve and boxplot for CRP on day 2 (AUC=0,72; P=0,0003). Horizontal lines in interquartile box represent the median (lower part of the figure)

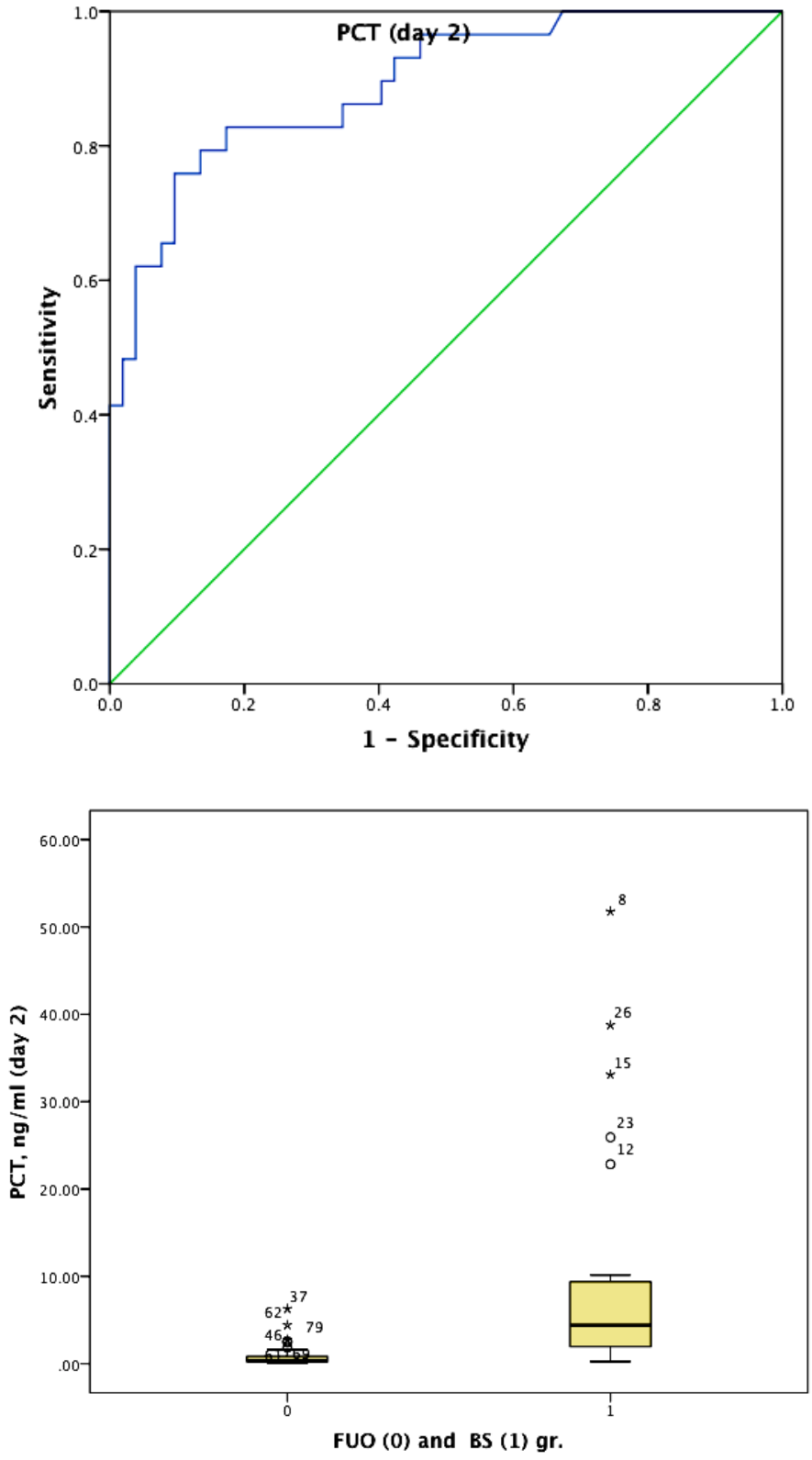


Figure 9. ROC curve and boxplot for PCT on day 2 (AUC=0,89; P<0,0001). Horizontal lines in interquartile box represent the median (lower part of the figure)

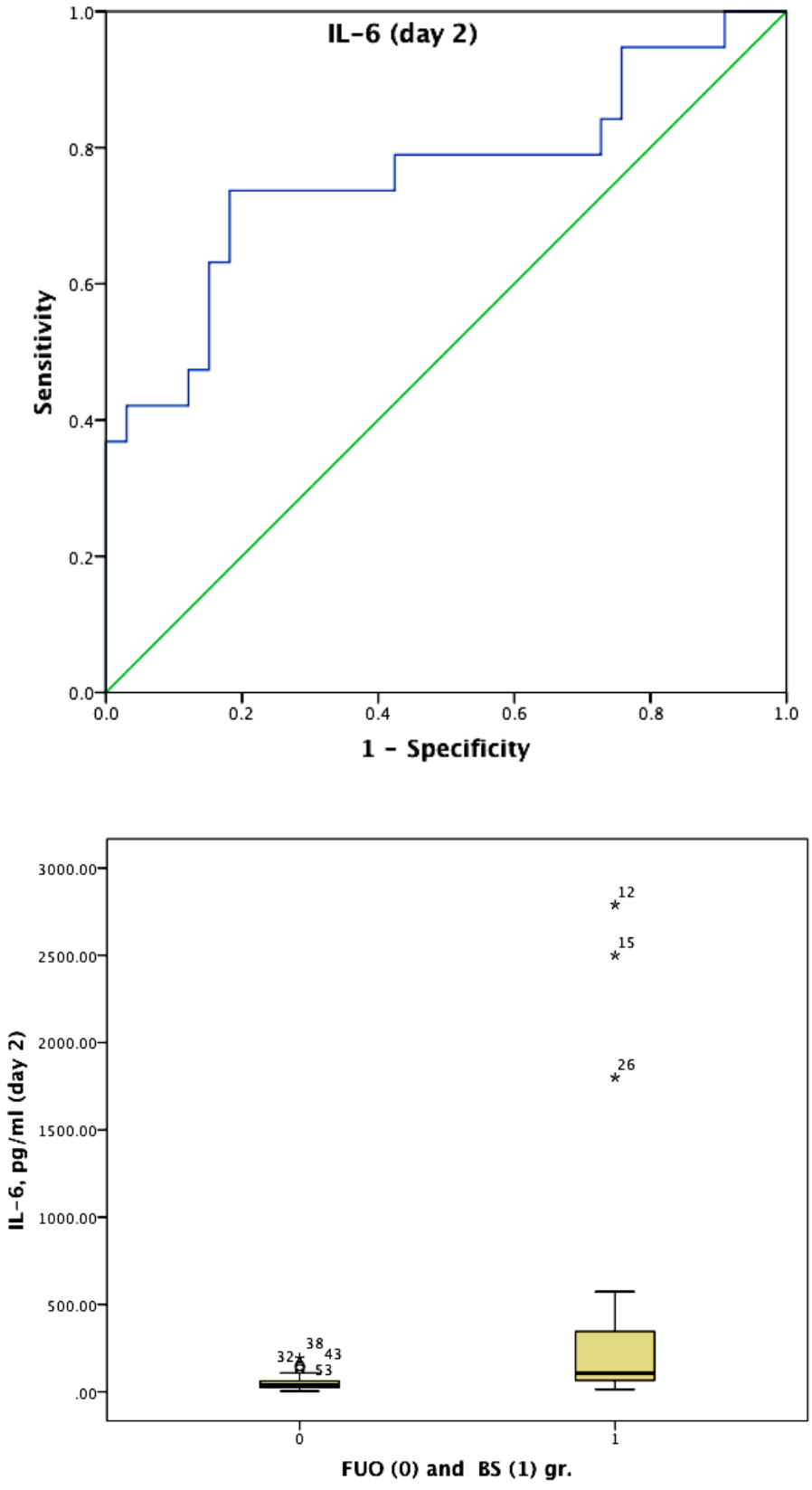


Figure 10. ROC curve and boxplot for IL-6 on day 2 (AUC=0,76; P=0,0003). Horizontal lines in interquartile box represent the median (lower part of the figure)

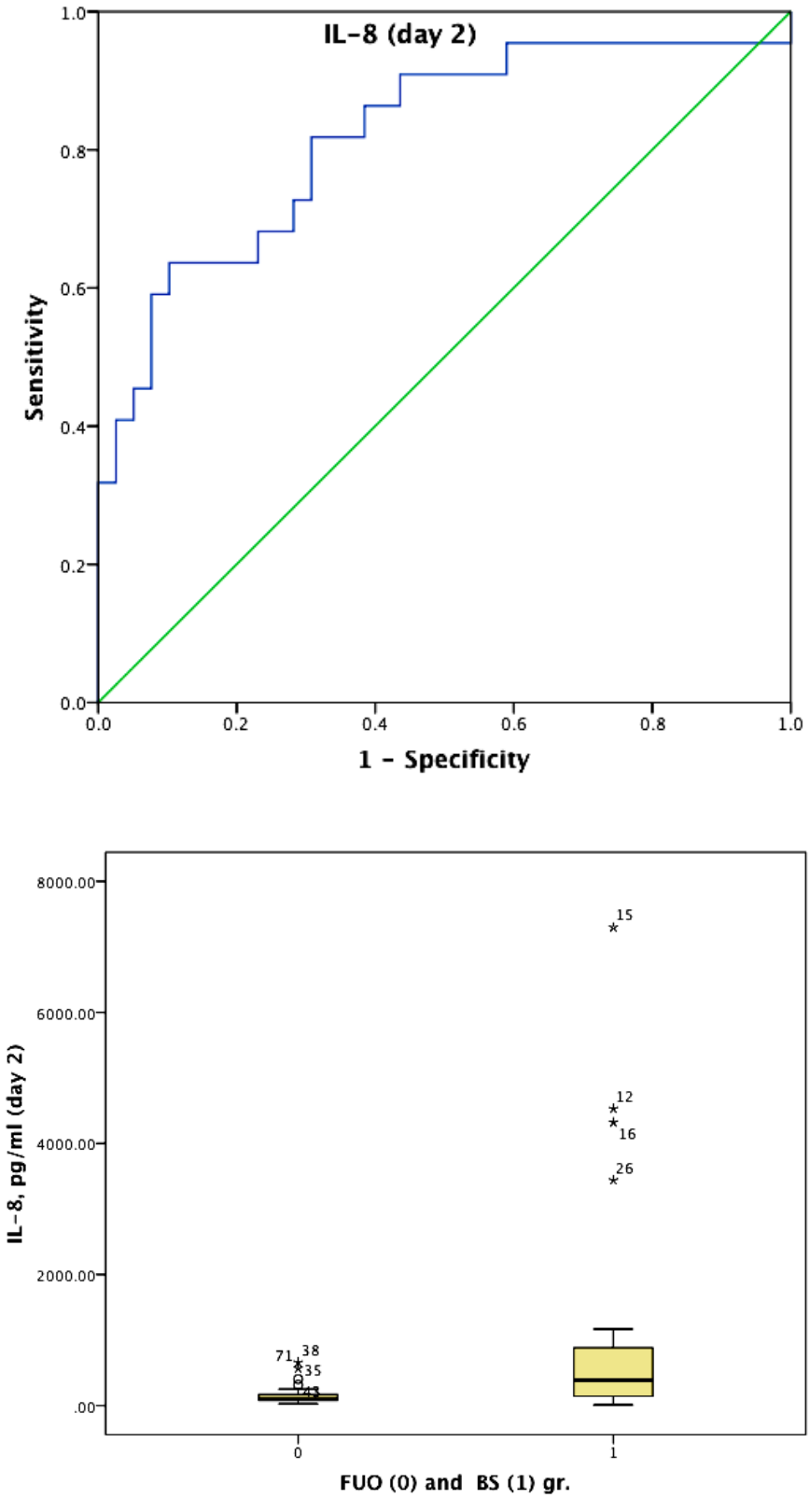


Figure 11. ROC curve and boxplot for IL-8 on day 2 (AUC=0,82; P<0,0001). Horizontal lines in interquartile box represent the median (lower part of the figure)

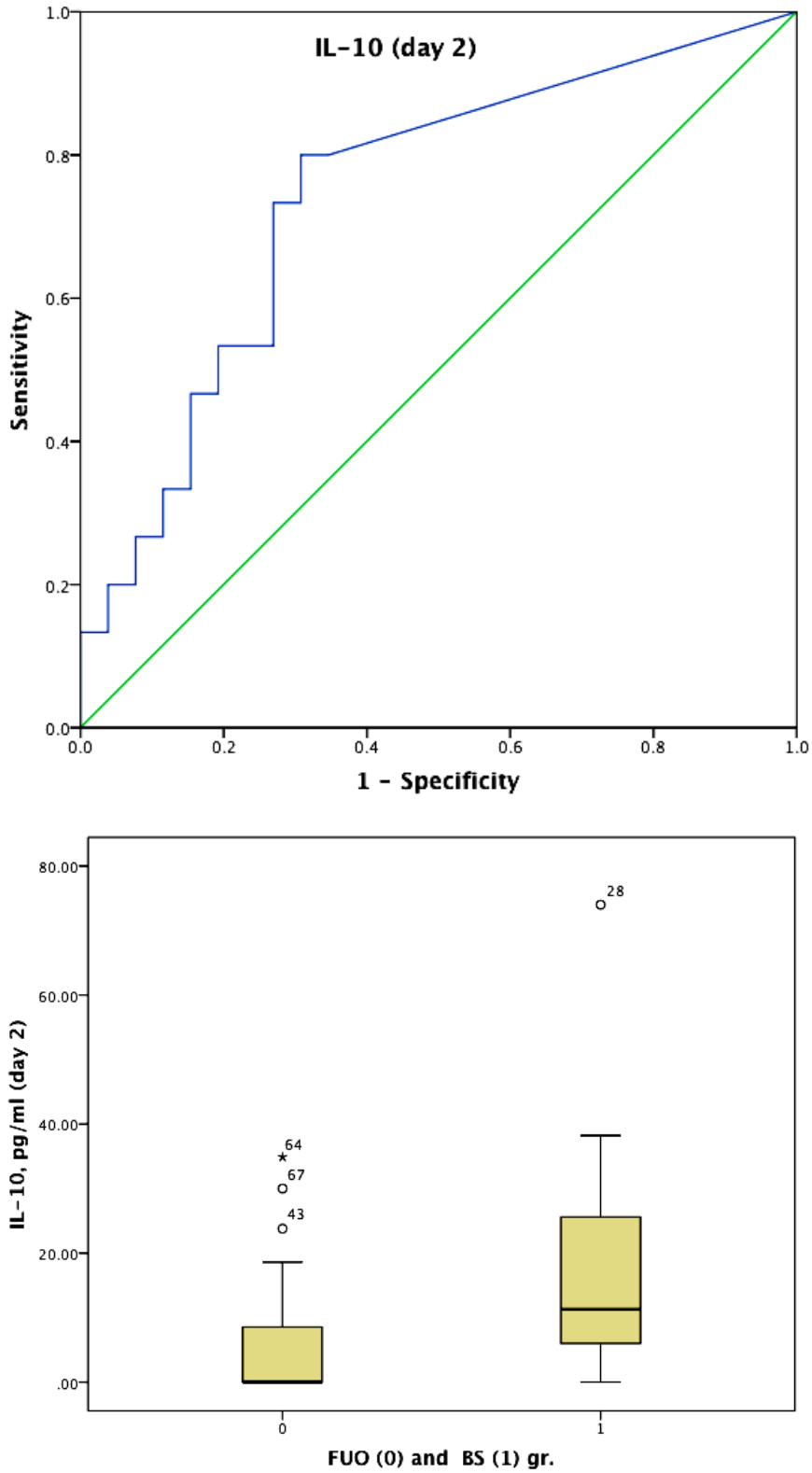


Figure 12. ROC curve and boxplot for IL-10 on day 2 (AUC=0,74; P=0,0041). Horizontal lines in interquartile box represent the median (lower part of the figure)

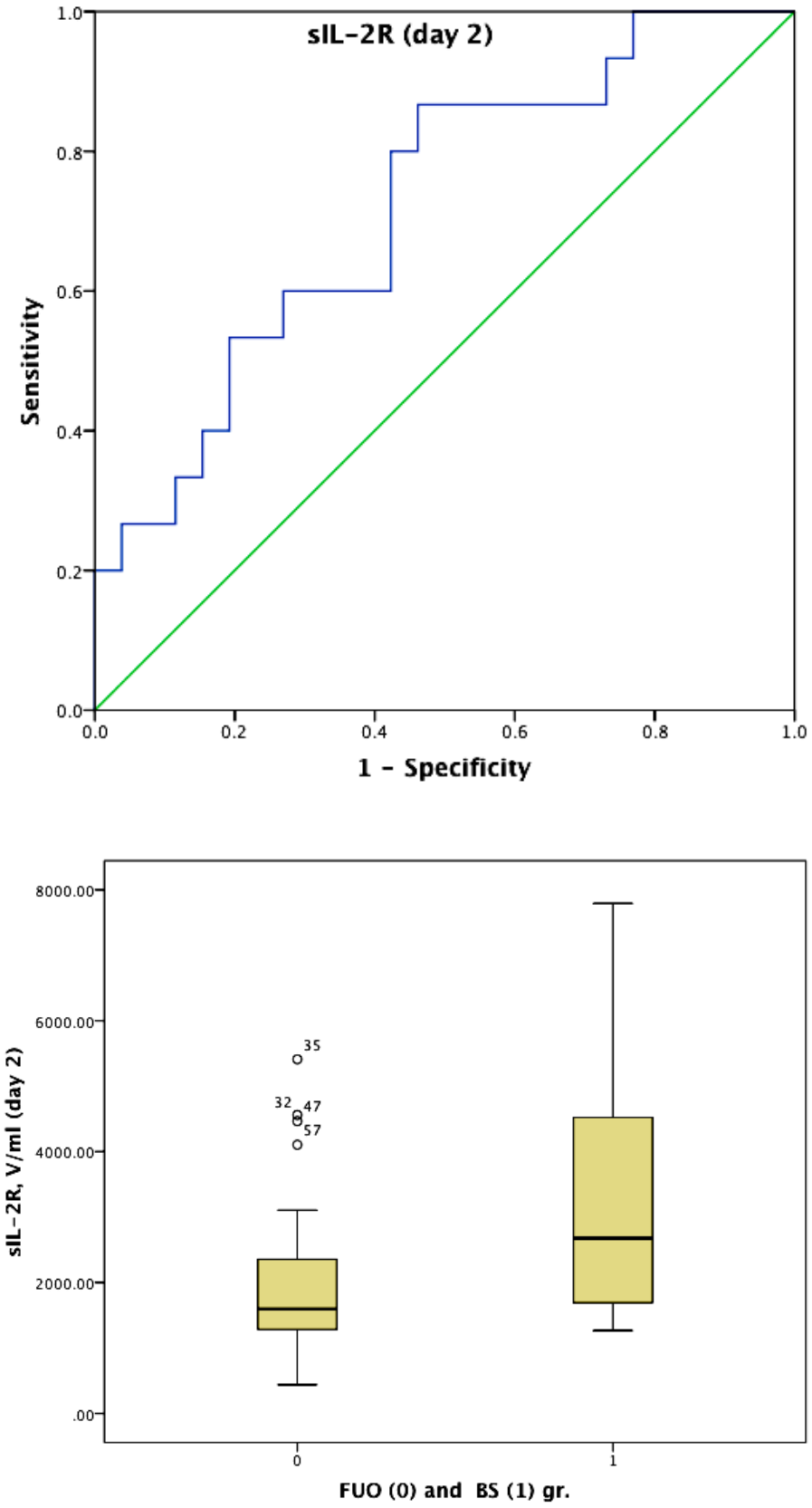


Figure 13. ROC curve and boxplot for sIL-2R on day 2 (AUC=0,72; P=0,0109). Horizontal lines in interquartile box represent the median (lower part of the figure)

The areas under the curves (AUC) for IL-8 and PCT on day 2 were above 0,80 (AUC_{IL-8}=0,82; AUC_{PCT}=0,89) suggesting that these two biomarkers discriminated bacteremia/sepsis with good accuracy. AUC of other evaluated biomarkers were lower than 0,80 and they were in the range of 0,72–0,76 (AUC_{IL-6}=0,76; AUC_{sIL-2R}=0,72; AUC_{CRB}=0,72; AUC_{IL-10}=0,74).

After assessing AUC changes of examined biomarkers during the first two consecutive days of FN, we can conclude that accuracy of IL-8, PCT, CRB increased (due to increase of AUC), whereas accuracy of IL-6, IL-10, sIL-2R decreased (due to decrease of AUC).

Sensitivity, specificity, negative and positive predictive values (NPV, PPV) for these biomarkers were also calculated and evaluated (Table 1).

Table 1. Diagnostic utility of cytokines and other biomarkers for identifying bacteremia/sepsis positive patients, according to receiver-operating characteristic (ROC) analysis.

Marker	Days	AUC	Cut-off	Sensitivity, %	Specificity, %	PPV, %	NPV, %
IL-6	Day 1	0,87	108 pg/ml	87	76	66	91
	Day 2	0,76	108 pg/ml	47	88	68	76
IL-8	Day 1	0,81	316 pg/ml	60	85	68	80
	Day 2	0,82	316 pg/ml	59	92	81	81
PCT	Day 1	0,79	1,13 ng/ml	38	98	91	75
	Day 2	0,89	1,13 ng/ml	83	83	72	90
IL-10	Day 1	0,82	11,3 pg/ml	67	78	61	81
	Day 2	0,74	11,3 pg/ml	47	81	57	74
sIL-2R	Day 1	0,74	1658 V/ml	79	69	58	86
	Day 2	0,72	1658 V/ml	80	58	51	84
CRP	Day 1	0,60	61 mg/l	38	69	40	67
	Day 2	0,72	61 mg/l	93	42	46	92

On day 1 for the evaluated biomarkers sensitivity was 38-87%, specificity was 69-98%, PPV was 40-91% and NPV was 67-91%, accordingly. On day 2 for

these biomarkers sensitivity was 47-93%, specificity was 42-92%, PPV was 46-81% and NPV was 74-92%.

Of note, highly specific tests have low false-positive rates, therefore specificity is one of the main characteristics of diagnostic biomarker, whereas for the monitoring purposes we should use highly sensitive tests due to their low false-negative rates. However, specificity reflects the intrinsic test validity and it is not related to the prevalence of certain disorders. Therefore, it is very important to calculate predictive values, which assess the validity of positive/negative tests in terms of presence/absence of pathologic conditions. Overall diagnostic biomarker should have high specificity and PPV, whereas biomarker dedicated to screening – high sensitivity and NPV.

On day 1 for the diagnostic purposes the most suitable among the investigated biomarkers was PCT with specificity 98% and PPV – 91% (cut-off was 1,13 ng/ml), whereas for the screening purposes the best among biomarkers was IL-6 (sensitivity was 87%, NPV – 91%, cut-off was 108 pg/ml). On day 2 the most suitable among evaluated molecules for the diagnostic purposes was IL-8 (specificity was 92%, PPV was 81%, cut-off – 316 pg/ml), in contrast for the screening purposes the most valuable was CRP (sensitivity was 93%, NPV – 92%, cut-off was 61 mg/l).

For the first time sHLA-G was assessed as a biomarker for bacteremia/sepsis evaluation. According to our results, on day 1 accuracy of sHLA-G was 0,72 (AUC=0,72; P=0,0274). This biomarker had more pronounced screening features with specificity of 73% and NPV – 83%.

Comparison of biomarkers concentration between investigated groups. On day 1 and day 2 medians of cytokines and other biomarkers levels were significantly higher in BS group compared to FUO group, except CRP on day 1, levels of which between investigated groups did not differ significantly. Table 2 shows detailed data on biomarkers concentrations in the blood of investigated patients on the first and second days respectively.

Table 2. Comparison of biomarkers concentrations in the investigated groups according to the Mann-Whitney test (IL-6, IL-8, IL-10 values were expressed in pg/ml, CRP in mg/l, sIL-2R and sHLA-G were expressed in U/ml).

Marker, day	Groups (FUO, BS)	Lower value	Higher value	Median	Median 95% CI	P
Day 1						
IL-6	FUO	18	558	65	39-93	<0,0001
	BS	54	1818	281	122-1081	
IL-8	FUO	18	855	140	81-211	0,0001
	BS	72	4227	636	198-1360	
IL-10	FUO	0	87	0	0-8	0,0017
	BS	0	1000	31	4-596	
sIL-2R	FUO	594	4012	1480	966-2026	0,0136
	BS	1054	7286	2342	1593-4307	
CRB	FUO	2	242	34	23-55	0,1256
	BS	5	140	40	28-91	
PCT	FUO	0,02	1,15	0,27	0,18-0,34	<0,0001
	BS	0,17	22	0,57	0,34-2,31	
sHLA-G	FUO	0	134	2,06	1,73-3,30	0,0410
	BS	0	119	4,76	1,88-38	
Day 2						
IL-6	FUO	4	193	38	29-61	0,0019
	BS	14	2790	105	52-395	
IL-8	FUO	27	658	98	86-139	<0,0001
	BS	9	7297	386	149-856	
IL-10	FUO	0	35	0	0-7	0,0105
	BS	0	74	11	3-29	
sIL-2R	FUO	440	5410	1595	1432-2229	0,0199
	BS	1265	7790	2675	1654-5297	
CRB	FUO	12	240	73	54-95	0,0010
	BS	10	197	122	90-142	
PCT	FUO	0,05	6,28	0,36	0,26-0,55	<0,0001
	BS	0,26	52	4,38	2,07-8,92	

On day 1 median of IL-6 in FUO group was 65 pg/ml and in BS group was 281 pg/ml, respectively ($p<0,0001$). Median of IL-8 in FUO group was 140 pg/ml and in BS group 636 pg/ml, accordingly ($p=0,0001$). Median of IL-10 was 0 pg/ml, whereas in BS group was 31 pg/ml ($p=0,017$). Median of sIL-2R in FUO was 1480 pg/ml, in contrast to BS group – 2342 pg/ml ($p=0,0136$). Median of PCT value in FUO was 0,27 ng/ml and in BS group was 0,57 ng/ml ($p<0,0001$) and median of sHLA-G in FUO was 2,06 U/ml, whereas in BS group was 4,76 U/ml ($p=0,0410$). Medians of CRP did not differ significantly ($p=0,0410$) and in FUO and BS groups were 34 mg/l and 40 mg/l, accordingly.

On day 2 median of IL-6 in FUO group was 38 pg/ml and in BS group was 105 pg/ml, respectively (p=0,0019). Median of IL-8 in FUO group was 98 pg/ml and in BS group 386 pg/ml, accordingly (p<0,0001). Median of IL-10 was 0 pg/ml, whereas in BS group was 11 pg/ml (p=0,0105). Median of sIL-2R in FUO was 1595 pg/ml, in contrast to BS group – 2675 pg/ml (p=0,0199). Median of PCT value in FUO was 0,36 ng/ml and in BS group was 4,38 ng/ml (p<0,0001) and median of CRP in FUO was 73 mg/l, whereas in BS group was 122 mg/l (p=0,0010).

Changes of biomarkers concentration in bacteremia/sepsis group during the first three consecutive days of febrile neutropenia. Changes of biomarkers concentrations in BS group were analyzed according to one-way analysis of variance (ANOVA) and *Post Hoc* analysis. During the first three days downward trend was observed in cytokines (IL-6, IL-8, IL-10) concentration changes. Decrease of IL-6 and IL-8 concentrations was not statistically significant, whereas IL-10 levels decline during the first two days was statistically significant (P = 0.009, Bonferroni post hoc analysis), however, during the second and third day only downward trend of concentration variations was observed. In contrast, sIL-2R concentration during the first three consecutive days increased, however, this increase was not statistically significant. Concentrations of CRP and PCT during the first two days increased and these variations were statistically significant (P<0,001 and P=0,035, accordingly, Bonferroni *Post Hoc* analysis), however, on the second and third day concentration of these biomarkers decreased and these changes were not statistically significant.

Relationship between confirmed bacteremia/sepsis and values of investigated biomarkers. Binary logistic regression analysis was used to describe the relationship between concentrations of biomarkers and microbiologically or clinically established bacteremia/sepsis. Models of multiple biomarkers (ranged from two to seven), created using above mentioned method further were evaluated by receiver-operating characteristic (ROC) analysis and according to AUC accuracy along with ability to differentiate between BS and FUO groups were assessed (Figures 14, 15).

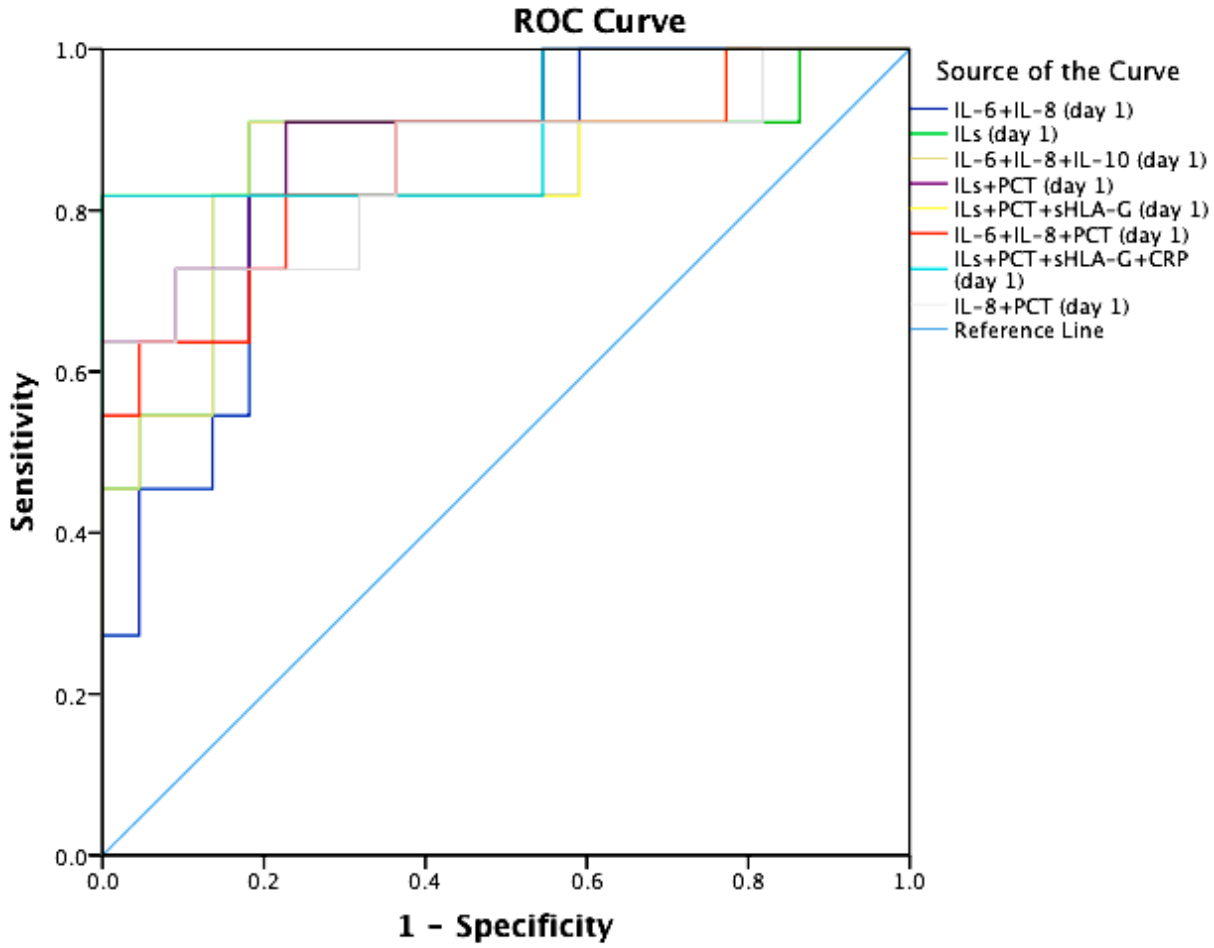


Figure 14. Set of biomarkers accuracy for sepsis/bacteremia evaluation according to ROC analysis on day 1.
$AUC_{IL\text{-}6+IL\text{-}8}$=0,82; $AUC_{IL\text{-}6+IL\text{-}8+IL\text{-}10}$=0,87; AUC_{ILs}=0,86;
$AUC_{ILs+PCT}$=0,91; $AUC_{ILs+PCT+sHLA\text{-}G}$=0,88; $AUC_{ILs+PCT+sHLAG+CRP}$=0,90;
$AUC_{IL\text{-}6+IL\text{-}8+PCT}$=0,86; $AUC_{IL\text{-}8+PCT}$=0,86 (ILs means IL-6+IL-8+IL-10+sIL-2R).

On day 1 the most accurate was set of five biomarkers composed of IL-6, IL-8, IL-10, sIL-2R and PCT ($AUC_{ILs+PCT}$ =0,91), while other arrays of biomarkers consisted of six (IL-6, IL-8, IL-10, sIL-2R, PCT, sHLA-G) and seven (IL-6, IL-8, IL-10, sIL-2R, PCT, sHLA-G) biomarkers had lower accuracy (AUC=0,88 and AUC=0,90, respectively) for sepsis/bacteremia discrimination. Sufficiently high accuracy had investigated arrays composed of two and three biomarkers ($AUC_{IL\text{-}6+IL\text{-}8+PCT}$=0,86; $AUC_{IL\text{-}8+PCT}$=0,86; $AUC_{IL\text{-}6+IL\text{-}8+IL\text{-}10}$=0,87). Pearson correlation coefficient was used to evaluate correlation between the most accurate array consisting of IL-6, IL-8, IL-10, sIL-2R and PCT and the array composed of two

biomarkers (PCT and IL-8). According to evaluation results, correlation between these sets of biomarkers was strong (r_s = 0,90; p<0,0001).

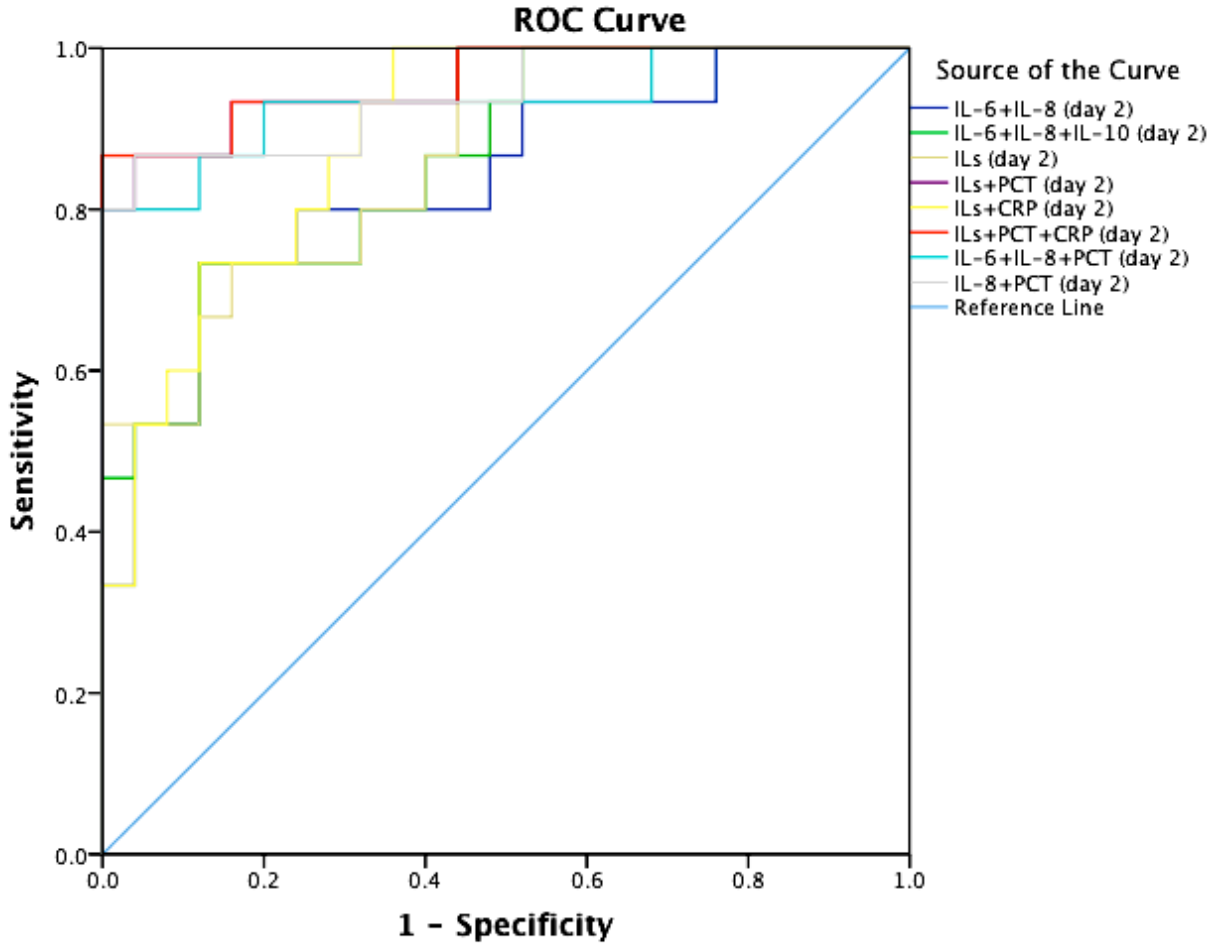


Figure 15. Set of biomarkers accuracy for sepsis/bacteremia evaluation according to ROC analysis on day 2.
$AUC_{IL\text{-}6+IL\text{-}8}$=0,84; $AUC_{IL\text{-}6+IL\text{-}8+IL\text{-}10}$=0,86; $AUC_{IL\text{-}ai}$=0,86; $AUC_{IL\text{-}ai+CRB}$=0,89; $AUC_{IL\text{-}ai+PCT}$=0,96; $AUC_{ILai+PCT+CRB}$=0,96; $AUC_{IL\text{-}6+IL\text{-}8+PCT}$=0,93; $AUC_{IL\text{-}8+PCT}$=0,94 (ILs means IL-6+IL-8+IL-10+sIL-2R).

On day 2 the most accurate were sets of five (IL-6, IL-8, IL-10, sIL-2R, PCT) and six (IL-6, IL-8, IL-10, sIL-2R, PCT, CRP) biomarkers with accuracy of 0,96 ($AUC_{IL\text{-}ai+PCT}$=0,96; $AUC_{ILai+PCT+CRB}$=0,96). Other arrays composed of 2-5 biomarkers had accuracy in range of 0,84–0,94 (AUC=0,84–0,94). Pearson correlation coefficient was used to evaluate correlation between the most accurate array consisting of IL-6, IL-8, IL-10, sIL-2R and PCT and the array

composed of two biomarkers (PCT and IL-8). According to evaluation results, correlation between these sets of biomarkers was strong (r_s = 0,93; p<0,0001).

Conclusions

1. On day 1 the most accurate biomarkers for bacteremia/sepsis discrimination were cytokines such as IL-6 (AUC=0,87), IL-10 (AUC=0,82) and IL-8 (AUC=0,81), on day 2 – IL-8 (AUC=0,82) and PCT (AUC=0,89);
2. On day 1 the most expressed diagnostic features had PCT (specificity was 98%, PPV – 91%), while for screening purposes the best biomarker was IL-6 (sensitivity was 87%, NPV – 91%). On day 2 of febrile neutropenia the best biomarker for diagnostic was IL-8 (with specificity of 92% and PPV – 81%), whereas for screening purposes was CRP (with sensitivity of 93% and NPV – 92%);
3. Usage of biomarkers arrays increases the accuracy of bacteremia/sepsis discrimination (on day 1 +0,04; $AUC_{IL\text{-}6}$=0,87 vs $AUC_{ILs+PCT}$=0,91, on day 2 +0,07; AUC_{PCT}=0,89 vs $AUC_{ILs+PCT}$=0,96);
4. Evaluated biomarkers were not suitable for monitoring purposes (concentration changes during the first three consecutive days were not statistically significant).

List of publications

1. V. Urbonas, A. Eidukaitė, I. Tamulienė, L. Ragelienė, S. Burokienė, J. Raistenckis, V. Tamošiūnas. **Value of interleukins in pediatric oncohematology patients with febrile neutropenia for the assessment of low risk bacteremia group** *(in lithuanian). Medicinos teorija ir praktika* 2010; 16(2):117-123.
2. Urbonas V, Eidukaitė A, Tamulienė I. **The Diagnostic Value of Interleukin-6 and Interleukin-8 for Early Prediction of Bacteremia and Sepsis in Children With Febrile Neutropenia and Cancer.** *J Pediatr Hematol Oncol* 2012; 34:122–127.

3. Urbonas V, Eidukaitė A, Tamulienė I. **Increased interleukin-10 levels correlate with bacteremia and sepsis in febrile neutropenia pediatric oncology patients**. *Cytokine* 2012; 57: 313–315.
4. Urbonas V, Eidukaitė A, Tamulienė I. **The predictive value of soluble biomarkers (CD14 subtype, interleukin-2receptor, human leucocyte antigen-G) and procalcitonin in the detection of bacteremia and sepsis in pediatric oncology patients with chemotherapy-induced febrile neutropenia**. *Cytokine* 2013; 62: 34-37.

Participation at conferences, abstracts

1. Tamulienė I, Urbonas V, Eidukaitė A, Ragelienė L. **Determination a group with low risk of bacterial infection among children with cancer and febrile neutropenia**. Poster presentation at **7th Baltic Conference of Hematology"**, May 20-22, 2010, Tartu, Estonia.
2. Urbonas V, Eidukaite A, Tamuliene I. **Calprotectin (S100A8/A9) is not predictive of bacteremia and sepsis in febrile neutropenia pediatric oncology patients.** Poster presentation at **"20th IFCC-EFLM European Congress of Clinical Chemistry and Laboratory Medicine (EuromedLab)"**, May 19-23, 2013, Milan, Italy.

Abstracts:

1. Urbonas V, Eidukaitė A, Tamulienė I**. Interleukin-8 Values in Pediatric Oncology Patients with Febrile Neutropenia and Bloodstream Infections**. *Pediatric Research* 2011; 70: 490–490; doi:10.1203/01.pdr.0000403893.61640.b6.

Reziumė

Pastaraisiais metais taikant pažangius bei modernius gydymo metodus ženkliai padidėjo onkologinėmis ligomis sergančių vaikų išgyvenamumas, kuris šiuo metu siekia apie 75%. Šie vaikai dažniausiai miršta dėl pagrindinės savo ligos, t.y. onkologinio proceso organizme, tačiau apie 16% mirties atvejų sudaro taikomo intensyvaus gydymo komplikacijos. Viena iš pagrindinių taikomos intensyvios chemoterapijos komplikacijų yra organizmo imuninės sistemos slopinimas ir su tuo susijusi neutropenija, kuri savo ruožtu sąlygoja padidėjusią riziką susirgti bakterinės kilmės infekcinėmis ligomis. Tarp onkologinėmis ligomis sergančių vaikų, karščiavimas bei neutropenija yra dažniausi ir neretai vieninteliai gresiančios bakterinės infekcijos požymiai gydymui taikomos intensyvios chemoterapijos fone. Kadangi bakterinio pobūdžio susirgimas tarp šių ligonių gali komplikuotis sepsiu bei sepsiniu šoku, visiems be išimties pacientams, kuriems nustatomas karščiavimas bei ženklus neutrofilų kiekio sumažėjimas kraujyje, taikomas intensyvus gydymas intraveniniais plataus spektro antibiotikais ir šie pacientai yra guldomi į ligoninę intensyviam sekimui. Ši gydymo taktika, taikoma febrilinės neutropenijos (FN) metu, ženkliai sumažino mirtingumą, susijusį su infekcinio pobūdžio komplikacijomis. Tačiau net 70–89% pacientų su febriline neutropenija infekcijos sukėlėjai taikant mikrobiologinius metodus nenustatomi. Tai rodo, kad didžioji dalis vaikų su FN galėtų būti priskiriama žemos bakterinės kilmės komplikacijų rizikos grupei, kuriai būtų taikomi alternatyvūs gydymo metodai bei ambulatorinė priežiūra, tuo būdu išvengiant stacionarinės priežiūros ir su tuo susijusios gyvenimo kokybės blogėjimo, intraveninių antibiotikų vartojimo bei rezistentiškos mikrofloros išsivystymo, pavojaus užsikrėsti antibiotikams atsparia hospitaline infekcija bei išlaidų, susijusių su gydymu bei vaikų priežiūrai skirtu tėvų nedarbingumu. Mažesnei daliai vaikų su komplikuota (bakteriemija, sepsis) FN eiga turėtų būti taikomas agresyvus aukščiau paminėtas gydymas antibiotikais bei stacionarinė priežiūra (aukštos bakterinės kilmės komplikacijų rizikos grupė). Todėl labai svarbu kasdienėje klinikinėje praktikoje turėti jautrius bei specifinius diagnostinius, atrankinius bei riziką vertinančius biožymenis, kurių pagalba būtų galima vaikus su FN priskirti žemai ar aukštai bakterinės kilmės komplikacijų

rizikos grupei bei anksti diagnozuoti beprasidedantį infekcinį procesą, tuo būdu išvengiant nereikalingo plataus spektro intraveninių antibiotikų vartojimo, stacionarinės priežiūros, gyvenimo kokybės blogėjimo bei papildomų išlaidų.

Šio darbo tikslas buvo įvertinti ūmaus bakterinio uždegimo bei sepsio patogenezėje dalyvaujančių citokinų (IL-6, IL-8, IL-10), citokinų receptorių (sIL-2R), ūmios fazės baltymų bei kitų imuninio atsako komponentų (CRB, PCT, sHLA-G) tinkamumą bakterinio proceso ankstyvai diagnostikai tarp pacientų su FN, šių biožymenų tinkamumą ir pritaikomumą kasdienėje klinikinėje praktikoje.

Tiriamoji medžiaga surinkta 2009 – 2011 m. Vilniaus universiteto Vaikų ligoninės Onkohematologijos skyriuje. Į tyrimą buvo įtraukta 53 onkohematologinėmis ligomis sergantys vaikai su FN, kurie gydymo eigoje turėjo 82 karščiavimo epizodus. Tyrimai buvo atliekami Inovatyvios medicinos centre bei Vilniaus universiteto Vaikų ligoninės Laboratorinės diagnostikos skyriuje. Tyrimui atlikti gautas Lietuvos bioetikos komiteto leidimas Nr. 158200-12-130-35 (2009-12-02). Nuo pirmos karščiavimo dienos tris dienas iš eilės buvo imami kraujo mėginiai bei nustatomos citokinų (IL-6, IL-8, IL-10), CRB, PCT ir sIL-2R koncentracijos. sHLA-G koncentracija buvo tirta tik pirmą karščiavimo dieną. Kraujo pasėliai aerobams, anaerobams, grybelinei kultūrai buvo imami pirmą karščiavimo parą prieš paskiriant antibakterinį gydymą. Remiantis klinikinių bei mikrobiologinių tyrimų duomenimis, FN epizodai buvo suskirstyti į dvi grupes – neaiškios kilmės karščiavimo (NKK), į kurią buvo įtraukti pacientai be sepsio požymių bei su neigiamais mikrobiologiniais pasėliais ir bakteriemijos-sepsio (BS). BS grupę sudarė pacientai su teigiamais mikrobiologiniais pasėliais ir(ar) kliniškai diagnozuotu sepsiu. 10 tirtų pacientų mirė. 18 pacientų turėjo daugiau nei vieną FN epizodą (po 2 epizodus turėjo vienuolika pacientų, po 3 – keturi pacientai, po 4 – vienas pacientas, po 5 – vienas pacientas, po 6 – vienas pacientas). Pacientų amžius svyravo nuo 1 iki 17 metų (mediana – 6 metai), tyrime dalyvavo 23 moteriškos lyties ir 30 vyriškos lyties pacientų. BS tiriamųjų grupę sudarė 22 pacientų (8 moteriškos lyties bei 14 vyriškos lyties), kurie tiriamojo periodo metu turėjo 29 karščiavimo epizodus (du pacientai turėjo po 2 karščiavimo epizodus, vienas – turėjo 3 epizodus ir vienas – 4). Teigiami mikrobiologiniai pasėliai buvo nustatyti 23 FN epizodų metu, sepsis – 6 FN

epizodų metu. Gram-neigiami mikroorganizmai buvo nustatyti 12 FN epizodų metu, vyraujant *Esherichia coli* sukeltai infekcijai. Gram-teigiami mikroorganizmai buvo mikrobiologiškai nustatyti 8 FN epizodų metu, vyraujant stafilokokinei mikroflorai, o trimis atvejais buvo nustatyta mišri mikroflora. Bakteriemijos-sepsio dažnis tirtoje imtyje sudarė 35% ir šis rodiklis neprieštarauja kitų tyrėjų rezultatams, kuriuose teigiama, kad bakterinio pobūdžio karščiavimas neutropenijos metu sudarė 10-45% visų FN atvejų (Castagnola et al., 2007; Bakhshi et al., 2008; Klastersky et al., 2007; Vellenga et al., 1996; Klastersky et al., 2000). Mūsų atliktame tyrime tirtų biožymenų (IL-6, IL-8, IL-10, sIL-2R, PCT, CRB, sHLA-G) savybes identifikuoti bakterinę infekciją bei įvertinti jos riziką analizavome pirmą ir antrą FN epizodo dieną (pirmosios valandos kliniškai yra reikšmingiausios, nes gydytojas hematologas turi priimti sprendimą dėl gydymo taktikos pasirinkimo). Mes nustatėme, kad pirmą ir antrą FN epizodo dienomis IL-6, IL-8 ir PCT koncentracijų medianos reikšmingai skyrėsi tarp NKK ir BS grupių. CRB koncentracijų mediana tarp nagrinėjamų grupių pirmą dieną statistiškai reikšmingai nesiskyrė ir statistinį reikšmingumą įgijo tik antrą FN epizodo dieną ir tai neprieštarauja kitų autorių (Miedema et al., 2011; Kalio et al., 2001) duomenims, kuriuose CRB tampa statistiškai reikšmingas vėliau - antrą-trečią FN epizodų dienomis. Šių biožymenų tikslumas pirmą dieną svyravo 0,60-0,87 intervale (AUC=0,60-0,87). Labiausiai išreikštu gebėjimu diferencijuoti NKK ir BS grupes bei didžiausiu tikslumu pasižymėjo IL-6 (AUC=0,87), kai tuo tarpu IL-8 ir PCT tikslumas buvo mažesnis ir praktiškai vienodame lygyje (AUC_{IL-8}=0,81; AUC_{PCT}=0,79), o CRB siekė tik 0,60 (AUC=0,60). IL-10 tikslumas siekė 0,82 (AUC=0,82), o sIL-2R diagnostinis tikslumas buvo 0,74 (AUC=0,74). Antrą FN epizodo dieną tiksliausias biožymuo buvo PCT ir siekė 0,89 (AUC_{PCT}=0,89), o kitų vertintų biožymenų tikslumas svyravo 0,72-0,82 ribose (AUC_{IL-6}=0,76; AUC_{IL-8}=0,82; AUC_{CRB}=0,72). sIL-2R ir IL-10 AUC reikšmės skyrėsi viena nuo kitos labai nedideliame intervale ir atitinkamai buvo 0,72 (AUC_{sIL-2R}=0,72) ir 0,74 (AUC_{IL-10}=0,74). Diagnostinėmis savybėmis pasižymintis biožymuo privalo turėti pakankmai didelį specifiškumą bei TNV, kai tuo tarpu atrankinio pobūdžio – didelį jautrumą bei NNV. Pirmą FN epizodo dieną labiausiai išreikštomis diagnostinėmis savybėmis tarp IL-6, IL-8 ir

PCT pasižymėjo PCT biožymuo, kurio specifiškumas buvo 98%, o TNV – 91% (ribinei vertei esant 1,13 ng/ml), kai tuo tarpu atrankiniam tyrimui labiausiai tiko – IL-6 (jautrumas – 87%, NNV – 91%). Antrą dieną tinkamiausiu diagnostikai tarp šių biožymenų buvo IL-8 (specifiškumui esant 92%, o TNV – 81%), o atrankiniam tyrimui – PCT (jautrumas buvo 83%, NNV – 90%, ribinei vertei esant 1,13 ng/ml). Analizavome ir kitų biožymenų, t.y. sIL-2R, IL-10 ir CRB savybes identifikuoti bakterinę infekciją bei įvertinti jos riziką. Šių biožymenų koncentracijų pokyčius vertinome pirmą ir antrą FN epizodo dieną ir nustatėme, kad pirmomis FN epizodo dienomis sIL-2R ir IL-10 koncentracijų medianos reikšmingai skyrėsi tarp NKK ir BS grupių, o CRB skaitinių verčių medianos įgijo statistinį reikšmingumą tik antrą FN epizodo dieną. Kaip ir su prieš tai minėtais biožymenimis, atlikome šių biožymenų ribinių verčių suvienodinimą pagal ROC kreivių analizės duomenis. Pirmą bei antrą FN epizodo dieną labiau išreikštomis atrankinėmis savybėmis pasižymėjo sIL-2R biožymuo, kurio jautrumas buvo 79-80%, o NNV – 84-86%, kai tuo tarpu IL-10 jautrumas svyravo 47-67% ribose, o NNV – 74-81% tarpe. Diagnostinės šių biožymenų savybės buvo išreikštos silpniau, nes abiejų tirtų analičių TNV variavo pakankamai žemose ribose (TNV – 51-61%). Labiausiai išreikštomis atrankinėmis savybėmis antrą FN epizodo dieną pasižymėjo CRB, kurio jautrumas buvo 93%, o NNV – 92% (ribinei vertei esant 61 mg/l). Pirmą kartą sHLA-G buvo vertinamas ir analizuojamas kaip potencialus bakteriemijos bei sepsio indentifikavimo biožymuo. Mūsų tyrimų duomenimis, pirmą FN epizodo dieną sHLA-G koncentracijų medianos reikšmingai skyrėsi tarp NKK ir BS grupių, o tikslumas siekė 0,72 (AUC=0,72). Šis biožymuo pasižymėjo labiau išreikštomis atrankinėmis savybėmis t.y. bakteriemijos-sepsio atmetimui (neigiamas rezultatas), ribinei vertei esant 2,54 V/ml (jautrumas buvo 73%, NNV – 83%). Taikant binarinę logistinę regresiją, buvo sukurti modeliai, kuriuos sudarė nuo 2 iki 7 biožymenų paletės, kurie toliau buvo analizuojami taikant ROC kreivių metodą. Didžiausiu gebėjimu diferenciuoti NKK ir BS grupes pirmą bei antrą dieną pasižymėjo diagnostinė paletė, kurią sudarė citokinai, citokinų tirpūs receptoriai ir PCT (5 biožymenų paletė), kurių AUC atitinkamai buvo 0,91 (pirmą dieną), o antrą dieną siekė net 0,96. Palyginus šių palečių ir atitinkamų pavienių citokinų AUC reikšmes pirmą ir antrą

dieną, galime daryti išvadą, kad taikydami paletes galime pasiekti didesnį tikslumą, kuris pirmą dieną būtų +0,04 (AUC_{IL-6}=0,87; $AUC_{ILs+PCT}$=0,91), o antrą dieną būtų +0,07 (AUC_{PCT}=0,89; $AUC_{ILs+PCT}$=0,96).

Išvados:

- Bakteriemijos-sepsio vertinimui FN epizodų metu tiksliausi biožymenys citokinų, citokinų receptorių grupėje pirmą FN epizodo dieną buvo IL-6 (AUC=0,87), IL-10 (AUC=0,82) ir IL-8 (AUC=0,81), antrą FN epizodo dieną – IL-8 (AUC=0,82) ir IL-6 (AUC=0,76);
- Bakteriemijos-sepsio vertinimui FN epizodų metu tiksliausias biožymuo tarp kitų uždegimo patogenezėje dalyvaujančių biomolekulių buvo PCT, kurio AUC pirmą bei antrą dienomis atitinkamai buvo 0,79 ir 0,89;
- Pirmą FN epizodo dieną labiausiai išreikštomis diagnostinėmis savybėmis pasižymėjo PCT biožymuo, kurio specifiškumas buvo 98%, o TNV – 91%, kai tuo tarpu atrankiniam tyrimui labiausiai tiko – IL-6 (jautrumas – 87%, NNV – 91%). Antrą dieną tinkamiausiu diagnostikai buvo IL-8 (specifiškumas buvo 92%, o TNV – 81%), o atrankiniam tyrimui – CRB (jautrumas buvo 93%, NNV – 92%);
- Bakteriemijos-sepsio vertinimui FN epizodų metu naudojant biožymenų paletes tikslumas didėja, t.y. pirmą dieną +0,04 (AUC_{IL-6}=0,87; $AUC_{ILs+PCT}$=0,91), o antrą dieną +0,07 (AUC_{PCT}=0,89; $AUC_{ILs+PCT}$=0,96);
- Sekimui analizuoti citokinai, citokinų tirpūs receptoriai, CRB ir PCR netiko, nes jų koncentracijos pokyčiai pirmą-trečią FN epizodo dienomis nebuvo statistiškai reikšmingi, tačiau buvo galima įžvelgti šių biožymenų pokyčių tendencijas, t.y. IL-6, IL-8, IL-10 koncentracijos mažėjo; CRB, PCT koncentracijos pirmą-antrą dieną didėjo, trečią dieną mažėjo; sIL-2R koncentracija didėjo.

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